The Hedgehog (Hh) signaling pathway is intimately linked to cell growth and differentiation, with normal roles in embryonic pattern formation and adult tissue homeostasis and pathological roles in tumor initiation and growth. Recent advances in our understanding of Hh response have resulted from the identification of new pathway components and new mechanisms of action for old pathway components. The most striking new finding is that signal transmission from membrane to cytoplasm proceeds through recruitment, by the seven-transmembrane protein Smoothened, of an atypical kinesin, which routes pathway activation by interaction with other components of a complex that includes the latent zinc finger transcription factor, Ci.

The expression and activity of Hedgehog proteins (Hh) exemplify a common strategy for pattern generation in metazoan embryos, namely, the specification of multiple cell fates through localized production and secretion of an instructive signal. In this manner, Hh signals regulate cell proliferation and differentiation in a diverse array of essential patterning events ranging from embryonic segmentation and appendage development in insects (Fig. 1A) to neural tube differentiation in vertebrates (1). But, Hh signaling also assumes homeostatic roles in postembryonic tissues to maintain stem cells (2–6), and continuous Hh pathway activity plays a pathological role in the growth of a group of endodermally derived human cancers that together account for ~25% of human cancer deaths (7–9). Despite the importance of these processes in human health and disease, substantial gaps remain in our understanding of the mechanisms that mediate Hh signal response. We review here recent fundamental advances in this area.

**Pathway Overview**

Hh proteins enter the secretory pathway and undergo autoprocessing and lipid modification reactions that produce a signaling peptide dually modified at its N- and C-termini by palmitoyl and cholesteryl adducts, respectively (10). Despite its dual lipid modification and consequent tight association with membranes, the Hh protein acts directly on distant cells in developing tissues. This remote action requires the transmembrane transporter-like protein Dispatched (Disp) for release of Hh from secreting cells (11–14), the heparan sulfate proteoglycans Dally-like (Dlp) and Dally for extracellular transport of Hh protein (15), and enzymes such as Sulfateless and Tout velu that are required for heparan sulfate biosynthesis (16), also an apparent transmembrane transporter that in the absence of Hh acts catalytically to suppress activity of the seven-transmembrane protein Smoothened [Smo (19)] (Fig. 1B). Inactivation of Ptc by binding to Hh permits activation of Smo, which in turn results in activation of latent cytoplasmic transcription factors, the Ci protein in *Drosophila* and the homologous Gli proteins in mammals. These aspects of the Hh signal response circuitry are well established and widely conserved from insects to mammals. Many of these findings, however, have their basis in genetic studies, which do not provide a mechanistic understanding of how the Hh signal is sensed, how Ptc switches Smo activity off, and how activation is routed from Smo in the membrane to Ci or Gli in the cytoplasm. Our consideration of these issues centers in great part on *Drosophila*, where recent advances have identified new components in-

**Fig. 1. Hedgehog signal response.** (A) *Drosophila* genetic systems used in the study of Hh signaling. Hh expressed in the posterior (P) compartment of the embryonic segment or the wing imaginal disc (green) induces transcriptional activation of target genes in a graded fashion in the corresponding anterior (A) compartments (red). We focus on Hh signal response as elucidated by loss-of-function studies using traditional mutational approaches in the embryo or imaginal disc or by disruption of gene expression using RNAi (RNA-mediated interference). (B) Overview of Hh pathway response. Autoprocessed, dually lipidated Hh protein released from producing cells binds to and inactivates Ptc, thus permitting activation of Smo in the responding cell. Activated Smo transmits a signal to the Ci transcriptional effector, which induces target gene expression.

**Transcriptional Repression or Activation in Hh Response**

Hh-responsive changes in gene expression are mediated by the zinc finger transcription factor
Ci, which can assume repressing and activating forms. The repressing form, CiR, comprises an N-terminal proteolytic fragment that retains the zinc finger–mediated DNA binding specificity of Ci but lacks nuclear export signals, a cytoplasmic anchoring sequence, and a transcriptional activation domain (20–22) (Fig. 2, A and B). Hh stimulation blocks CiR formation and causes increased nuclear import of cytoplasmic Ci, revealed inhibition of nuclear export by leptomycin B (LMB) (20, 22, 23). For some genes, such as decapentaplegic (dpp) in the wing imaginal disc, loss of CiR alone suffices for activation of expression, presumably because of the presence of an otherwise constitutively active promoter (24) (Fig. 2B). For other genes, such as the universal Hh pathway target ptc, expression requires not only loss of CiR but also the positive action of Ci (25). The expression of Hh pathway targets thus depends on regulation of Ci processing and localization.

**Proteolytic Processing of Ci to Form CiR**

Formation of CiR requires phosphorylation of specific serine-threonine residues by cyclic adenosine monophosphate (cAMP)–dependent protein kinase [PKA (26)]. Action of PKA primes Ci for further phosphorylation by glycogen synthase kinase 3β [GSK3β (27, 28)] and a member of the CK1 family of kinases (27, 29) (Fig. 2, A and B). The phosphorylated form of Ci appears to be a substrate for a proteolytic processing reaction that requires function of the proteasome and of Slimb (Slmb), an F-box–containing E3 ubiquitin ligase component (30, 31). Direct ubiquitination of Ci and cleavage by the proteasome to form CiR, however, have been difficult to demonstrate, and their contributions to CiR formation thus could be indirect.

Neither these kinases (PKA, GSK3β, and CK1), the proteasome, nor Slmb are dedicated exclusively to Hh signaling. Their roles in Ci processing likely result from channeling of their activities to Ci by Costal-2 (Cos2), a kinesin-like protein that stably associates with Ci and is required for Ci processing (32–34) (Fig. 2A). Ci phosphorylation and processing may be mediated by Cos2 scaffolding of kinases with Ci, although direct associations of these kinases with Cos2 or Ci have not yet been reported.

**Smo Switches Ci Function by Recruitment of Cos2**

CiR formation appears to be regulated by interaction of Cos2 with Smo (35, 36) (Fig. 3). On stimulation of cells by Hh, the Smo protein is stabilized and accumulates at least 10-fold (34, 37, 38) (Fig. 3B). Cos2 is recruited to Smo via sequences in the Smo cytoplasmic tail [SmoC (34, 36, 39, 40)], resulting in a loss of Ci processing (35, 36). Indeed, overexpression of SmoC in a myristoylated, membrane-tethered form (myrSmoC) suffices for loss of CiR, as indicated by accumulation of uncleaved Ci and activation of dpp expression (35, 36) (Fig. 3C). Loss of CiR alone, however, is insufficient to activate the full range of pathway response, because expression of ptc and other target genes is not induced either by overexpression of myr-SmoC (35, 36) or by loss of components required for CiR formation, such as Slmb or GSK3β (27, 28, 30, 31).

In addition to promoting CiR formation, Cos2 also regulates Ci by anchoring it in the cytoplasm (22, 41–43). Thus, in cells or tissues treated with LMB, nuclear accumulation of Ci is observed upon loss of Cos2 activity. Nuclear accumulation of Ci similarly results from Hh stimulation or overexpression of myrSmoC, indicating that cytoplasmic anchoring activity of Cos2, like its role in CiR formation, is abrogated upon recruitment by Smo (35, 36).

**Cos2 as a Molecular Router That Channels Smo Activity**

Although loss of Cos2 produces some pathway activity, the highest levels of Hh response actually require the positive input of Cos2 (23, 41). One aspect of positive regulation by Cos2 is its absolute requirement for stability of the Fused (Fu) serine-threonine kinase, which may relate to a tight association between these two proteins (34). Hh-induced stabilization of Smo thus results in recruitment of both Fu and Cos2 (34, 40). A hint as to the function of Fu in pathway activation is its dispensability upon loss of Su(fu) [Suppressor of fused (44)], which exerts a negative regulatory effect on Ci [ (42), see below]. Dispensability of Fu with the loss of Su(fu) suggests a dedicated function for Fu in Su(fu) inactivation, possibly by direct phosphorylation (34).
The activated Smo switch thus is coupled to Cos2, which then routes the Hh signal by ceasing production of CiR and cytoplasmic anchorage of Ci, but also by inactivating Su(fu) through Fu (Fig. 3). Loss of Cos2 thus produces incomplete pathway activation by lifting CiR repression and Ci anchorage but without activating Fu. Consistent with this model, all Hh targets are activated upon loss of Cos2 if Su(fu) function is additionally removed (41). Similarly, the subset of Hh targets activated by overexpression of myr-SmoC is extended to include ptc upon additional loss of Su(fu) (36).

In addition to stabilizing Fu and mediating forward signaling events that affect Ci, Cos2 is also required for the stabilization of activated Smo, a critical aspect of a full response to the Hh signal (34). Cos2 thus functions not just to route the Hh signal forward to cytoplasmic components, but also in feedback amplification of the incoming signal through accumulation of activated Smo in the membrane.

Membrane Sensors of the Hh Signal

The activation state of the Smo switch is controlled at the membrane by a series of sensors, most immediately the Ptc protein. Studies in mammalian cells have demonstrated that Ptc regulates Smo activity indirectly and substoichiometrically. On the basis of primary sequence and predicted transmembrane topology, Ptc is a member of the resistance, modulation, division transporter family (19). These proteins export substrates across the bacterial membrane by a proton antiport mechanism, and their function requires a widely conserved motif in transmembrane span 4, which also is required for Ptc regulation of Smo (19). These structural and functional homologies suggest that Ptc may transport an endogenous molecule that modulates Smo activity, but such a molecule and Ptc transport activity have not yet been characterized (19). Another Drosophila membrane protein that appears to function as Hh sensors at or upstream of Ptc is Dlp, a member of the glypicam family of glycosylphosphatidylinositol-linked proteins (15, 29, 45). Dlp may bind directly to Hh (29, 46), suggesting a possible role in delivering Hh to Ptc (15, 29). Dlp is also involved in extracellular transport of the Hh signal for distant action (10, 15).

The Hh Response Network in Action

Several rapid changes in protein conformation or modification are elicited by Hh stimulation, including increased phosphorylation of Smo, Cos2, Fu, and Su(fu) (32–34, 38, 47) and decreased phosphorylation of Ci (22, 48). Although phosphorylation of Ci stimulates processing to form CiR, the residues targeted and the functional roles of phosphorylation of other pathway components are largely undefined. These changes in phosphorylation nevertheless may be functionally important, because they are regulated by Ptc and Smo and are triggered by Hh stimulation.

With these phosphorylation events as benchmarks, we can surmise that pathway activation begins within minutes after Hh stimulation (34, 47). These kinetics provide constraints in considering the physical mechanisms underlying pathway activation. Thus, for example, Ptc is proposed to function through transport of an endogenous molecule, perhaps a lipid, that modulates Smo activity (19). This cellular state, in which Smo is kept inactive, must dissipate rapidly upon Hh-mediated inactivation of Ptc. Changes in the subcellular distribution of this proposed modulator upon Hh-mediated loss of Ptc function thus should be consistent with the kinetics of pathway induction. Similarly, critical events proposed to activate Smo, such as changes in conformation (49), subcellular localization (38, 50), phosphorylation (38), or dimerization (35) must occur on a sufficiently rapid time scale to be consistent with the kinetics of pathway activation.

One of the primary functions of Smo upon activation would appear to be inhibition of Su(fu) activity through the activation of Fu (44). It is tempting to speculate that Su(fu) is inactivated by phosphorylation and that this results from Fu activation. However, only a small fraction of Su(fu) associates with the Cos2-Fu-Ci complex or is phosphorylated upon Hh stimulation, suggesting that phosphorylation may result from a transient interaction between Su(fu) and the Cos2-Fu-Ci complex (34). It is less clear, however, whether Smo activates Fu kinase through direct contact or indirectly via Cos2. The roles of phosphorylation and/or conformational shifts of Smo and Cos2 in activation of Fu are not yet established. The function of Su(fu) in curtailing Ci activity involves localization of Ci, either through cytoplasmic anchoring or nuclear export activity, and possibly suppression of Ci function in the nucleus (41, 42). Mammalian Su(fu) is capable of interacting with Gli proteins bound to DNA (51) and also interacts with chromatin modulating factors (52), suggesting that Su(fu) also could affect transcriptional activity by recruiting chromatin modulating factors to the DNA sites of Gli protein binding (52).

Speed, Precision, and Versatility of the Hh Response

Although rapid phosphorylation of pathway components upon Hh stimulation indicates a rapid initiation of Hh response, the overall kinetics of change in gene expression are considerably slower, because Hh-induced expression of transcriptional targets requires elimination of CiR, which in turn requires cessation of CiR production and degradation of existing CiR. CiR production is blocked by recruitment of the Cos2-Fu-Ci complex to Smo (34), which depends on accumulation of Smo through new protein synthesis (34, 38). The kinetics of Hh transcriptional

Fig. 3. Smo activation of target genes via Fu and Cos2. (A) In the absence of Hh stimulation, the action of Ptc keeps Smo in a state of inactivity and low abundance. Consequently, little cytoplasmic complex (Cos2-Fu-Ci) is recruited to Smo, Cos2 anchors Ci in the cytoplasm, and CiR formation proceeds unhindered. In addition, Fu is inactive and Su(fu) is able to exert its negative regulatory effect on Ci. The absence of Ci and the presence of CiR in the nucleus thus result in suppression of target gene activation. (B) Upon Hh stimulation, Smo is activated and accumulates, thereby recruiting most or all of the cytoplasmic complex. The consequent loss of the Cos2 anchor and CiR formation and the activation of Fu in transcriptional activation of the full range of transcriptional targets including dpp and ptc. (C) Overexpression of a myristoylated SmoC (myrSmoC) results in recruitment of the cytoplasmic complex but an inability to suppress Su(fu) function (35, 36). As a result, CiR formation is blocked and dpp expression is activated (Fig. 2). Unrestrained activity of Su(fu), however, prevents activation of target genes such as ptc.
Home box 9252 (Hedgehog, Hh) signaling has been implicated in axon guidance. Although Smo and Fz are related or identical components, including the Smoothened (Smo) and Frizzled (Fz) relatives of Smo, other mediators in other responses to Hh signaling cannot be ruled out, particularly in cytoplasmic or nuclear responses that do not involve transcriptional regulation via Ci and Gli. In this regard, it is notable that Wnt proteins, which signal via the Frizzled (Fz) relatives of Smo, also elicit a variety of responses that extend beyond transcriptional activation to include axonal guidance and Ca\(^{2+}\) mobilization (34).

**How Other Animals Respond to Hedgehogs**

The Hh pathway in other animals to some extent parallels that in *Drosophila*, albeit with duplications of certain pathway components, with substantial gaps in our understanding, and with pathway roles for some proteins that do not function in the *Drosophila* pathway (Fig. 4B). Some duplication appears simply to provide diverse tissue-specific patterns of expression (e.g., the three mammalian Hh proteins), whereas other duplications permit divergence of function, [e.g., the Gli homologs of Ci, which subdivide the positive (Gli2) and negative (Gli3) transcriptional regulatory functions of Ci among them (59, 60)].

Between Smo and the Gli proteins there is a considerable gap in our understanding of mammalian Hh signal response. The nearest homologs to Cos2 are not nearly as well conserved as Hh, Ptc, Smo, and Gli and have not been functionally linked to pathway regulation. Similarly, the putative Fz homolog shows more limited conservation (61), and its pathway role is not clear because loss of function analyses have not been reported. Somewhat surprisingly, given its relatively mild mutant phenotype in *Drosophila*, the best
conserved component known to act in the interval between Smo and Ci is Su(fu) (51). Its relatively high conservation in mammals suggests that it may play an important role in pathway regulation, and loss of function analyses using morpholino oligonucleotides in the zebrafish suggest that Su(fu) may indeed have pathway regulatory functions (62). In addition, several mammalian components recently identified in genetic screens appear to function between Smo and the Gli proteins, and some of these have orthologs that apparently do not contribute to Hh signaling in Drosophila [(63) and references therein]. The biochemical functions of these proteins in mammalian Hh signaling remain unknown.

The function of these novel pathway components in mammalian but not Drosophila Hh response is especially noteworthy in view of the high degree of functional conservation of other components of the Hh signal response network. Hedgehog-regulated expression of transcriptional targets across evolution thus is similarly responsive to the Ptc sensor and to the Smo switch and involves similar processing and/or activation of latent transcriptional regulators. But the unique roles of these novel mammalian components suggests that distinct mechanisms may be involved in routing signaling activity from Smo to Gli. The presence of a clear mammalian ortholog of Su(fu) and of a putative Fu ortholog suggest that these additional mechanisms may operate in tandem with some of the same routing mechanisms that operate in Drosophila, although the absence of a clear Cos2 ortholog also suggests that certain Drosophila routing mechanisms may have lesser importance in mammals. Further mechanistic investigation of signal routing from Smo to transcriptional effectors will be critical to an understanding of how evolution has shaped Hh signal response and of the manner in which this response can be disrupted to produce neoplasia or birth defects.

References and Notes
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